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ABSTRACT

Induced pluripotent stem (iPS) cells are derived from reprogrammed somatic cells and are similar to embryonic stem (ES) cells in morphology, gene/protein expression, and pluripotency. In this study, we explored the potential of iPS cells to differentiate into alveolar Type II (ATII)-like epithelial cells. Analysis using quantitative real time polymerase chain reaction and immunofluorescence staining showed that pulmonary surfactant proteins commonly expressed by ATII cells such as surfactant protein A (SPA), surfactant protein B (SPB), and surfactant protein C (SPC) were upregulated in the differentiated cells. Microphilopodia characteristics and lamellar bodies were observed by transmission electron microscopy and lipid deposits were verified by Nile Red and Periodic Acid Schiff staining. C3 complement protein, a specific feature of ATII cells, was present at high levels in culture supernatants demonstrating functionality of these cells in culture. These data show that the differentiated cells generated from iPS cells using a culture method developed previously (Rippon et al., 2006) are ATII-like cells.

To further characterize these ATII-like cells, we tested whether they could undergo epithelial to mesenchymal transition (EMT) by exposure to drugs that induce lung fibrosis in mice, such as bleomycin, and the combination of transforming growth factor beta1 (TGF_{b1}) and epidermal growth factor (EGF). When the ATII-like cells were exposed to either bleomycin or a TGF_{b1}–EGF cocktail, they underwent phenotypic changes including acquisition of a mesenchymal/fibroblastic morphology, upregulation of mesenchymal markers (Col1, Vim, a-Sma, and S100A4), and downregulation of surfactant proteins and E-cadherin.

We have shown that ATII-like cells can be derived from skin fibroblasts and that they respond to fibrotic stimuli. These cells provide a valuable tool for screening of agents that can potentially ameliorate or prevent diseases involving lung fibrosis.

1. Introduction

Epithelial to mesenchymal transition (EMT) is one of the processes in the body responsible for organogenesis and cellular plasticity giving rise to mesenchymal phenotypes such as fibroblasts and myofibroblasts from other types of mature cells. This transformation is characterized by loss of cell to cell contact marked by a decrease in cell adhesion protein E-cadherin (Kalluri and Neilson, 2003; Rippon et al., 2006) and a switch to N-cadherin (Brabletz et al., 1998), a change of morphology from a flattened epithelial cell type into a spindle-shaped, fibroblast-like appearance, and subsequent acquisition of cytoskeletal markers (S100A4, a-Sma, Vim, b-Catenin), extracellular matrix proteins (Col1, Col3, Fn1, Lama5), and transcription factors (Snai1, Snai2, Zeb1, Twist1, Lef-1, Ets-1, Gsc) (Kalluri and Neilson, 2003; Zeisberg and Neilson, 2009).

Several factors have been shown to induce EMT. Oncogenic pathways such as Ras, Wnt/b-Catenin, and Notch can induce EMT associated with repression of E-cadherin (Boyer et al., 1997). Environmental factors such as hypoxia and oxidative stress also contribute to the EMT phenotype (Manotham et al., 2004). Activation of the canonical Smad signaling pathway via tandem

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effects of transforming growth factor beta1 (TGF_{b1}) and epidermal growth factor (EGF) have been shown to be major mediators of EMT (Piek et al., 1999). Bleomycin, which acts by preventing incorporation of thymidine into the DNA, promotes EMT by inducing DNA strand breaks (Yamamoto et al., 2000). Serious complications of EMT in tissues involve impaired normal organ functions, and may eventually result in fibrotic lung diseases.

Embryonic stem cells (ESCs) are ideal for studying cell differentiation because of their unlimited self renewal property and pluripotency (Andrews, 2002; Bodnar et al., 2004; Ohtsuka and Dalton, 2008). Various laboratories have shown that alveolar epithelial cells can be derived from ESC *in vitro* (Coraux et al., 2005; Rippon et al., 2008; Wang et al., 2007a) but the use of ESC has major drawbacks such as immune rejection and ethical issues due to the utilization of embryonic tissues. In 2006, a method for retrodifferentiation of somatic cells to embryonic-like cells emerged by introduction of defined transcription factors (Takahashi and Yamanaka, 2006). These cells have indistinguishable properties to ESC such as morphology, gene expression profile, and pluripotency. Success in utilizing iPS cells in studying disease states has been presented by various laboratories including our own (Alipio et al., 2010; Hanna et al., 2007; Xu et al., 2009).

Here we demonstrate the ability to induce EMT in an *in vitro* cell culture system of ATII-like cells derived from iPS cells. These cells are useful tools in searching for new biomarkers of EMT or novel drugs that can prevent EMT.

2. Materials and methods

2.1. Generation of induced pluripotent stem (iPS) cells from murine tail-tip fibroblasts

Normal fibroblasts from the tail-tip of green fluorescent protein (GFP) transgenic mice [(C57BL/6-Tg(UBC-GFP) 30Scha/J Stock#004356, Jackson Laboratory, Bar Harbor, ME)] were transduced with four retroviruses encoding transcription factors Oct4, Sox2, Klf4, and C-Myc (purchased from Stemgent, San Diego, CA) and iPS cells were produced and characterized as previously described (Alipio et al., 2010; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007). Fourteen to twenty days post transduction, 10 iPS subclone colonies were picked and expanded. Two representative clones with similar morphology and intensity of GFP expression were used for alveolar Type II (ATII) epithelial cell differentiation. These clones exhibited all the characteristics of typical iPS cells: expression of transducing transcription factor mRNA as well as additional embryonic stem cell markers and the ability to form teratomas when injected subcutaneously into the flanks of nude mice, properties that we and others have reported previously (Alipio et al., 2010; Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007).

2.2. In vitro differentiation of iPS cells into alveolar Type II (ATII-like) epithelial cells

Alveolar Type II (ATII-like) cells were derived from iPS cells by a three-stage differentiation process, following previously described methods for murine ES cells with slight modifications (Rippon et al., 2006). The modifications include the following: (1) enhanced attachment of Embryoid Bodies (EBs) at Stage 2 by culture of EBs in media containing FBS instead of Knock Out Replacement Serum (KOSR), (2) improved viability of ATII-like epithelial cells by passaging the differentiating cells at Stage 2 Day 5 of alveolar cell differentiation instead of continuous culture for 11 days during the Stage 2 process, and (3) improved viability of ATII-like epithelial cells by supplementing the small airway growth medium with 10% FBS in Stage 3.

2.3. Epithelial to mesenchymal (EMT) induction of ATII cells

Stage 3, Day 7 ATII-like cells grown to 80% confluency were treated with bleomycin (APP Pharmaceuticals LLC, Schaumburg, IL) at 1, 2, 4, 8, and 16 μg/ml final concentration. Concurrently, the combined effects of TGF_{b1} and EGF on the Stage 3, Day 7 ATII-like cells were also assessed. We tested three doses of TGF_{b1} and EGF (both from Millipore Billerica, MA): 5 ng/ml TGF_{b1} + 50 ng/ml EGF, 10 ng/ml TGF_{b1} + 100 ng/ml EGF, and 20 ng/ml TGF_{b1} + 200 ng/ml EGF. The cells were exposed to either of the fibrosis-inducing treatments for 2, 4, and 7 days. Culture conditions were repeated at least three times in independent experiments.

2.4. Nile red

To assess lipid deposits, Stage 3 Day 7 ATII-like cells grown to 80% confluency were stained with Nile Red (Sigma, St. Louis, MO) according to the manufacturer's instruction.

2.5. PAS staining

To determine the presence of glycolipids, Stage 3 Day 7 ATII-like cells grown to 80% confluency were stained with Periodic Acid Schiff staining (EMD Chemicals Inc, Gibbstown, NJ) according to manufacturer's instruction.

2.6. Polymerase chain reaction (PCR)

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) and 2 μg of total RNA was reverse transcribed using cDNA Archive kit (Applied Biosystems, Foster City, CA). PCR reactions were ran using Platinum PCR Supermix High Fidelity (Invitrogen) and quantitative real time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Data was acquired using 7500 FAST Real Time PCR system (Applied Biosystems, Foster City, CA) and analyzed using Comparative CT method via SDS FAST System software version 1.4.0. Primer sequences for endogenous/total retroviral transcripts (Boyer et al., 1997; Kalluri and Neilson, 2003), the lung markers (Rippon et al., 2006), and other gene specific oligonucleotides (obtained from Roche's UniversalProbe library) are reported in the Supplementary Table S1.

2.7. Immunofluorescence staining

Immunofluorescence staining was performed on actively dividing cells cultured in 12 well plates as previously described (Alipio et al., 2010; Hanna et al., 2007; Xu et al., 2009). Primary antibodies diluted at 1:100 in 1% BSA were used for immunofluorescence staining: Oct4 and SSEA-1 (Chemicon, Billerica, MA), Sox2, Nanog, SPA, SPB, SPC, ABCA3, CC10, Cytochrome P450 A1A (all from Santa Cruz Biotechnology, Santa Cruz, CA), and Aquaporin 5 (Abcam, Cambridge, MA). The secondary antibodies used were goat anti-mouse PE (Sigma, St Louis, MO), goat anti-rabbit-PE and rabbit anti-goat-PE (both from Southern Biotech, Birmingham, Alabama). Nuclei were counterstained with 1 μg/ml 4′,6-diamidino-2-phenylindole (Sigma, St Louis, MO) for 10 min. Images were captured by QImaging camera (QImaging, Surrey, BC) and analyzed by QimagingPro software (QImaging, Surrey, BC).

2.8. Transmission electron microscopy (TEM)

One 100 mm semi-confluent tissue culture dish containing Stage 3 Day 7 ATII-like epithelial cells was trypsinized, the cells were washed with serum free medium containing 0.5% bovine serum albumin and centrifuged for 5 min at 300*g*. The cell pellet

was fixed with EM-grade 1X Karnovsky Fixative solution containing 2% Paraformaldehyde, 2.5% Glutaraldehyde and 0.1 M Sodium Phosphate Buffer (Electron Microscopy Sciences, Hatfield, PA) for 15 min. The cells were centrifuged, fresh fixative solution was added to the cell pellet, and the samples were packaged in between ice packs to be shipped to University of North Texas for further sample preparation. Once received, the fixed sample was washed three times with fresh 0.1 M sodium phosphate buffer. The samples were centrifuged and the cells were fixed with a second fixative solution of 2% OsO_4 (Electron Microscopy Sciences, Hatfield, PA) in water for 1 h at room temperature. The sample was washed again three times as previously described and dehydrated in a series of different ethanol concentrations. To ease infiltration of Epon into the cell pellet, the sample was treated with propylene oxide (Sigma, St. Louis, MO) two times for 15 min. The sample was then infiltrated at 1:1 with propylene oxide/Epon for 30 min, then at 1:3 ethanol/propylene oxide for 30 min, and finally at 100% Epon overnight. The following day, the sample was exposed to fresh Epon for 2 h before it was placed into a 70 °C oven for 18–24 h to polymerize the plastic. 70–100 nm thick sections were contrast stained with a saturated uranyl acetate solution (Sigma, St. Louis, MO) in 70% ethanol for 10 min, washed with deionized water, stained with Satos' lead citrate solution (Sigma, St. Louis, MO) for 1 min, washed with deionized water, and air dried. The stained sections were viewed on a Zeiss EM 910 TEM with 100 kV voltage. Images were captured on Kodak film SO-163, developed and scanned on a flatbed scanner to convert them into electronic files.

2.9. Western blot

Protein from Stage 3 Day 7 ATII-like cells (with and without EMT inducers) in 100 mm culture dish was quantified using EZQ® Protein Quantitation Kit (Invitrogen, Carlsbad, CA) in triplicate using an ovalbumin standard curve. Western blot analysis was performed as described (Wang et al., 2007b; Wishart et al., 2007). 30 μg of protein from each sample was loaded into NuPAGE® 4–12% Bis–Tris Gel (Invitrogen, Carlsbad, CA). The primary antibodies used were SPC, a-Sma, E-Cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), and b-Actin (LI-COR, Lincoln, NE), each at a 1:50 dilution. Secondary antibody dilution was 1:10,000 for donkey anti-goat IRDye 800CW, goat anti-rabbit IRDye 800CW, and goat anti-mouse IRDye 800CW (all from LI-COR, Lincoln, NE). Protein detection was performed using Odyssey Infrared Imaging System (LI-COR, Lincoln, NE).

2.10. Mouse C3 ELISA

The supernatant from Stage 3 Day 7 ATII-like cells was collected and tested for the presence of C3 protein. The quantification of Complement factor 3 (C3) was performed using a highly sensitive immunoassay Mouse C3 ELISA (Immunology Consultants Laboratory, Inc. Newberg, OR) according to manufacturer's instructions.

3. Results

Skin fibroblasts from GFP-C57BL6 mice were retrodifferentiated to iPS cells as previously described (Takahashi and Yamanaka, 2006), followed by a programmed induction of iPS cells to differentiate into alveolar Type II epithelial cells as previously described (Rippon et al., 2006) with minor modifications (see Section 2). Finally, the ATII-like cells were treated with fibrotic stimuli such as bleomycin or TGF_{b1}–EGF to create a fibrotic model *in vitro*.

3.1. Differentiation of iPS cells into alveolar Type II (ATII-like) epithelial cells

The three-stage protocol for the direct differentiation of iPS cells into ATII-like cells (see Section 2) generated flattened epithelial-like morphology (Fig. 1a) at Stage 3 Day 7. Electron microscopic analysis at this stage showed multiple lamellar bodies and microphilopodia, characteristics indicative of an ATII cell differentiation (Fig. 1b). mRNA expression of lung specific markers such as surfactant protein A (SPA), surfactant protein B (SPB), surfactant protein C (SPC, a Type II alveolar cell specific marker), and surfactant protein D (SPD), were upregulated at various time points during the final stage of the differentiation (Fig. 1c). There was an approximately 2.5 fold increase of SPC expression at Stage 3 Day 6 ATII-like cells compared to the fibroblast control, and the expression further increased at Stage 3 Day 16 ATII-like cells, suggesting that the cells persisted long term in this culture condition. This information is helpful for cell therapy studies involving lung progenitor transplantation for regenerative and injury repair. Clara cells (marked by CC10 gene) were also present and were upregulated at Stage 3 Day 6 but were markedly depleted by Stage 3 Day 16 differentiation, suggesting selective differentiation or accumulation of ATII-like cells (Fig. 1c). Protein expression analysis by immunofluorescence staining of Stage 3 Day 7 ATII-like cells showed positive staining for ABCA3 (marker for gene present in the lamellar bodies and involved in regulation of lipid transport and membrane trafficking), CC10 (marker for Clara cell), cytochrome P450 A1A (marker for alveolar epithelium), SPA (a pulmonary surfactant protein marker), SPB (a pulmonary surfactant protein marker), SPC (marker for ATII, a pulmonary surfactant protein marker), and Aquaporin 5 (marker for the major water channel expressed in Alveolar Type I cells in the lung) at Stage 3 Day 7 (Fig. 2a). Intracellular lipid deposits were detected in these cells by Nile Red fluorescence staining (Fig. 2b) and Periodic Acid Schiff (PAS) staining (Fig. 2c). MLE-12, a murine lung epithelial cell line, was used as the positive control for both lipid tests. ATII-like cells were also able to synthesize and secrete murine complement factor 3, which was detected at high levels relative to fibroblast control by ELISA, indicating successful generation of murine ATII-like cells that can provide pulmonary host defense and are able to produce an acute phase protein (Fig. 2d).

By morphology, transmission electron microscopy, gene expression, intracellular protein expression, Nile red and PAS staining, and the secretion of C3 strongly suggests that ATII-like cells have been generated *in vitro*.

3.2. ATII-like epithelial cells undergo epithelial to mesenchymal transition (EMT) by exposure to bleomycin

We initially determined the condition for the ATII-like cells to undergo EMT by titrating the bleomycin from 1 to 16 μg/ml and exposing the cells for 2, 4, and 7 days (data not shown). Induction of EMT was assessed by morphology, protein expression of SPC, Col1, and a-Sma, by quantitative real time PCR analysis (lung markers and EMT-related genes), and western blot (E-cadherin and a-Sma).

ATII-like cells responded to the treatment in a time and dose dependent manner. Because the cells carry the GFP gene, fluorescence images gave the best representation of the morphological changes. Two days of drug treatment gave only slight changes in morphology at the highest doses (8 and 16 μg/ml) while the untreated control maintained an epithelial phenotype. qRT-PCR analysis also showed very minimal upregulation of EMT markers with similar expression to the untreated control (data not shown). After 4 days of treatment, morphological evidence of EMT is observed

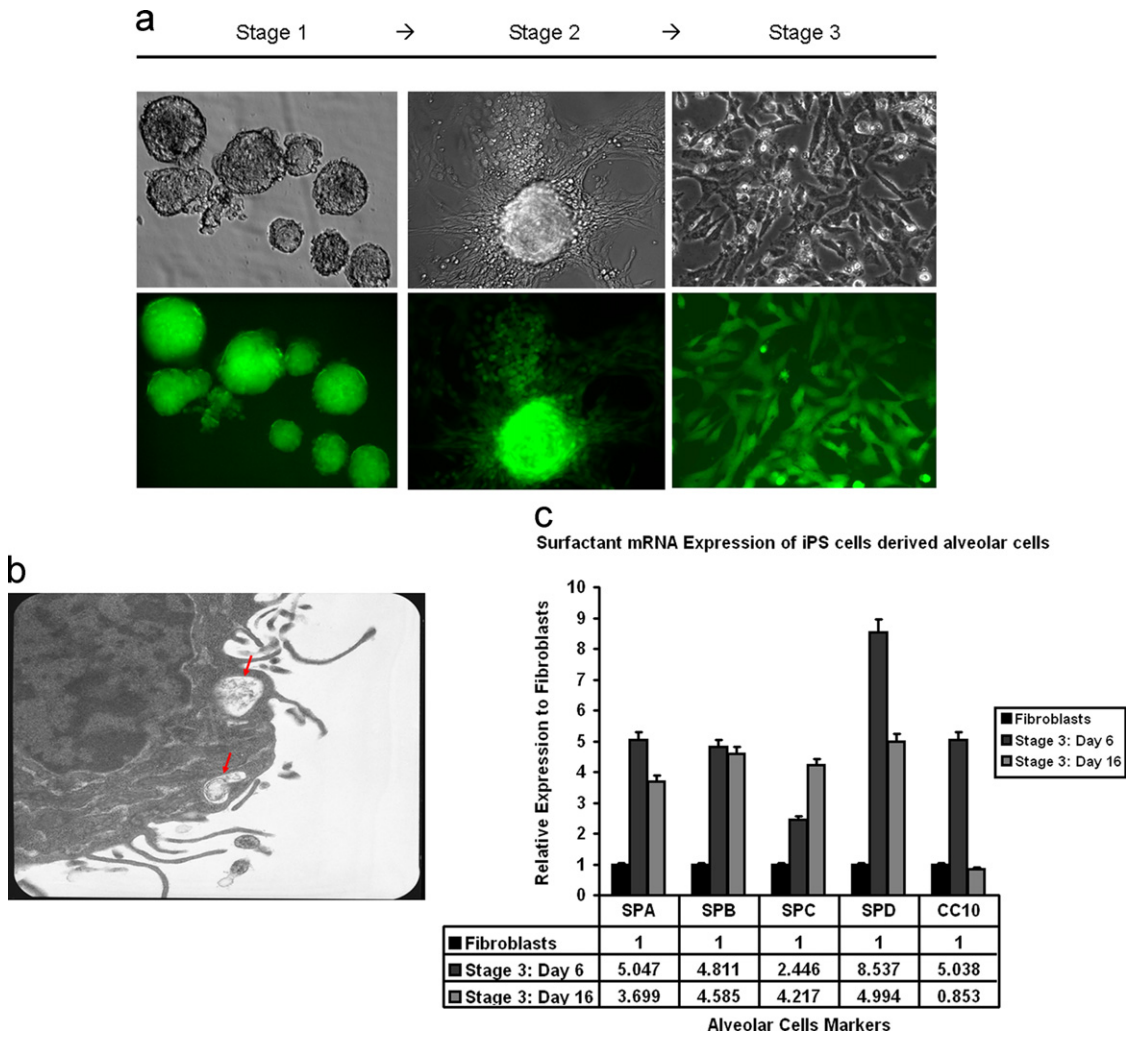

Fig. 1. (a) Morphology of the cells at each stage of the 3-step differentiation protocol (see Section 2). At Stage 1, iPS cells were differentiated into embryoid bodies (EBs), at Stage 2, EBs were attached to the tissue culture dish, and at Stage 3, a flattened epithelial-like cell phenotype was the prominent population grown in ATII selective media. (b) Transmission electron microscopy analysis of Stage 3 Day 7 cells showing microphilopodia (cytoplasmic extensions) and lamellar bodies (red arrows) typically seen in murine ATII cells. (c) Gene Expression of ATII markers (SPA, SPB, SPC, SPD) and Clara cells (CC10). All the ATII markers were upregulated at Stage 3 Day 6 and Day 16 of the differentiation while the Clara cell marker was only detected at the earlier time point indicating selected ATII differentiation/accumulation at the final stage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with cells adopting a fibroblast-like cell shape with extensive loss of cell to cell contact. This was observed in cells treated with 4 μg/ml of bleomycin treatment, and became more pronounced as the dose increased (Fig. 3a). Key EMT markers such as Snai1, Snai2 (Slug), Gsc, Cdh2, Col1, Mmp3, and Mmp9 were upregulated by qRT-PCR. Loss of E-cadherin, a hallmark of EMT, is also observed (Fig. 3b). With 7 days of treatment, a more extensive morphology change is observed even at a dose of 1 μg/ml bleomycin, (data not shown). More EMT markers were acquired at this dose and exposure time.

We then assessed the surfactant protein C (SPC) mRNA expression of the ATII-like cells after bleomycin treatment for 2, 4, and 7 days and showed that after 4 days of treatment, SPC is markedly downregulated by approximately 80% compared to the untreated control (Fig. 3b) starting at 2 μg/ml bleomycin. SPC protein expression was also analyzed at these time points by immunostaining (Fig. 3c) and showed SPC protein depletion as the dose of the bleomycin treatment increases in treated cells while high expression of SPC is observed in untreated control. E-cadherin is usually expressed very highly in ATII epithelial cells, and in this study, qRT-PCR (Fig. 3b) and western blot (Fig. 3d) analyses show that E-cadherin expression is also depleted as the concentration of the bleomycin treatment increases.

To further document that EMT is occurring in ATII-like cells, we analyzed gene expression of 52 EMT-related markers that were shown to be significantly upregulated during EMT by qRT-PCR (Kalluri and Neilson, 2003; Zeisberg and Neilson, 2009). We analyzed samples collected after 2, 4, and 7 days of bleomycin treatment at various doses. After 2 days of treatment, several EMT-related genes are upregulated relative to the untreated control group. The number of acquired markers continued to increase after 4 and 7 days of treatment, increasing from 13.5% to 36.5% positive hits (day 4) and 13.5% to 73.1% (day 7) in cells treated with 8 μg/ml bleomycin.

Many genes are related to EMT and its progression to trans-differentiation, including the switching of E-cadherin to N-cadherin. Although the E-cadherin to N-cadherin switch was not observed after 2 days, only marginally indicated at any bleomycin dose after 4 days of treatment, the switch was exhibited at all bleomycin doses after 7 days of treatment. Indeed E-cadherin expression was barely detectable after 7 days of treatment at all doses tested.

Upregulation of integrins are also linked to EMT since EMT disturbs the cell to fibrillar extracellular matrix interaction. The integrin 5 (integrin alpha 5, a fibronectin receptor) and Itgav

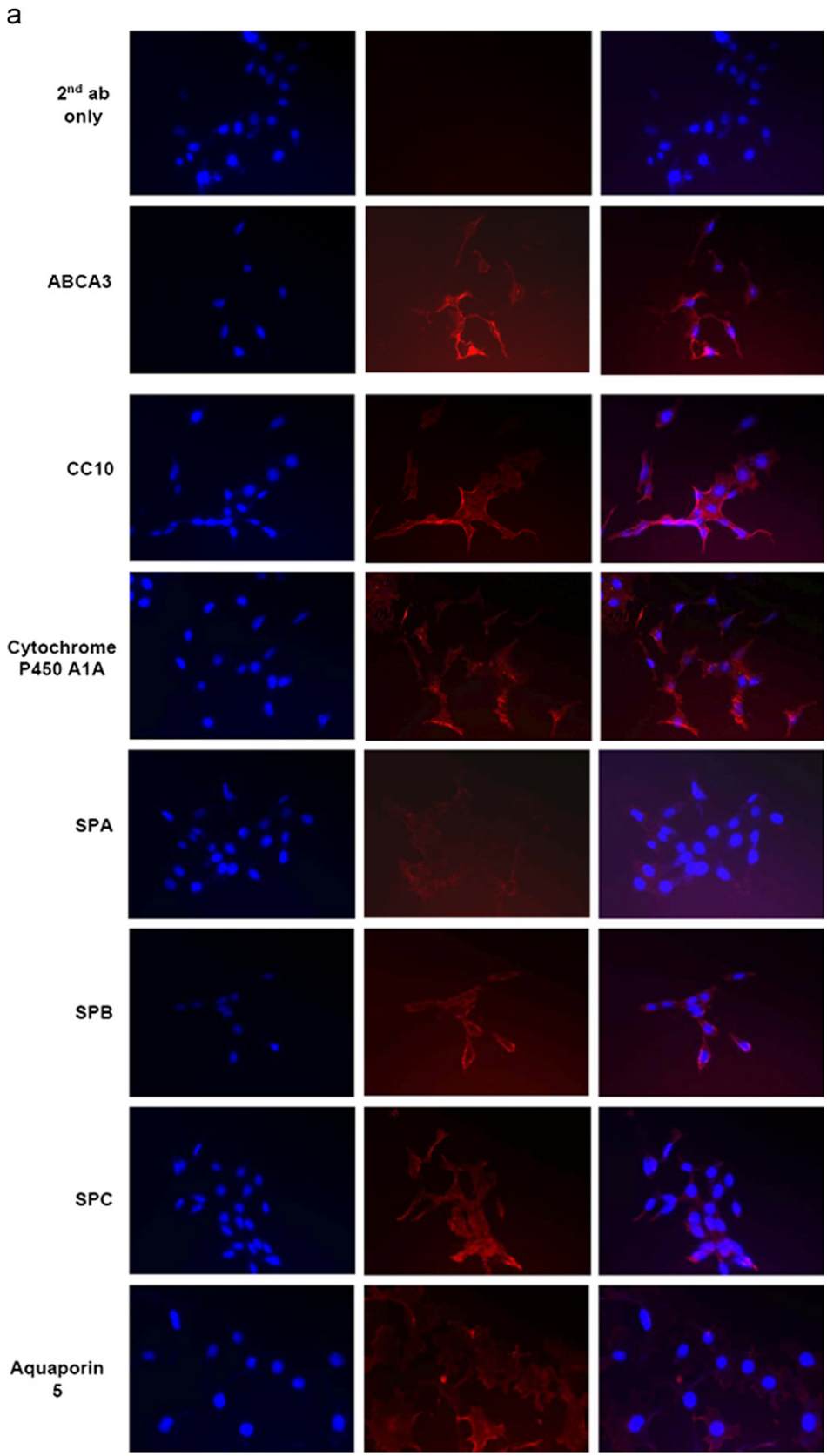

Fig. 2. (a) Protein expression analysis by immunofluorescence staining of Stage 3 Day 7 ATII-like cells showing positive staining for ABCA3 (a lamellar body marker), CC10 (a Clara cell marker), cytochrome P450 A1A, SPA, SPB, SPC (all alveolar markers), and aquaporin 5 (an AT Type I marker). All images were taken at 100 × magnification. (b) Nile Red staining showing lamellar bodies (cytoplasmic white fluorescence dots) in Stage 3 Day 7 ATII-like epithelial cells. The MLE epithelial cell line was used as positive control. Images were taken at 200 × magnification. (c) PAS staining showing positivity for the presence of lipid (red stain) in Stage 3 Day 7 ATII-like epithelial cells. MLE murine epithelial cell line was used as control. Images were taken at 200 × magnification. (d) Complement Factor 3 (C3) protein quantification in the supernatant of Stage 3 Day 6 and Day 16 ATII-like cells by an ELISA assay. Very high levels of C3 protein are secreted by the differentiated cells. Murine serum was used as the positive control and culture media from the fibroblasts was used as the negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

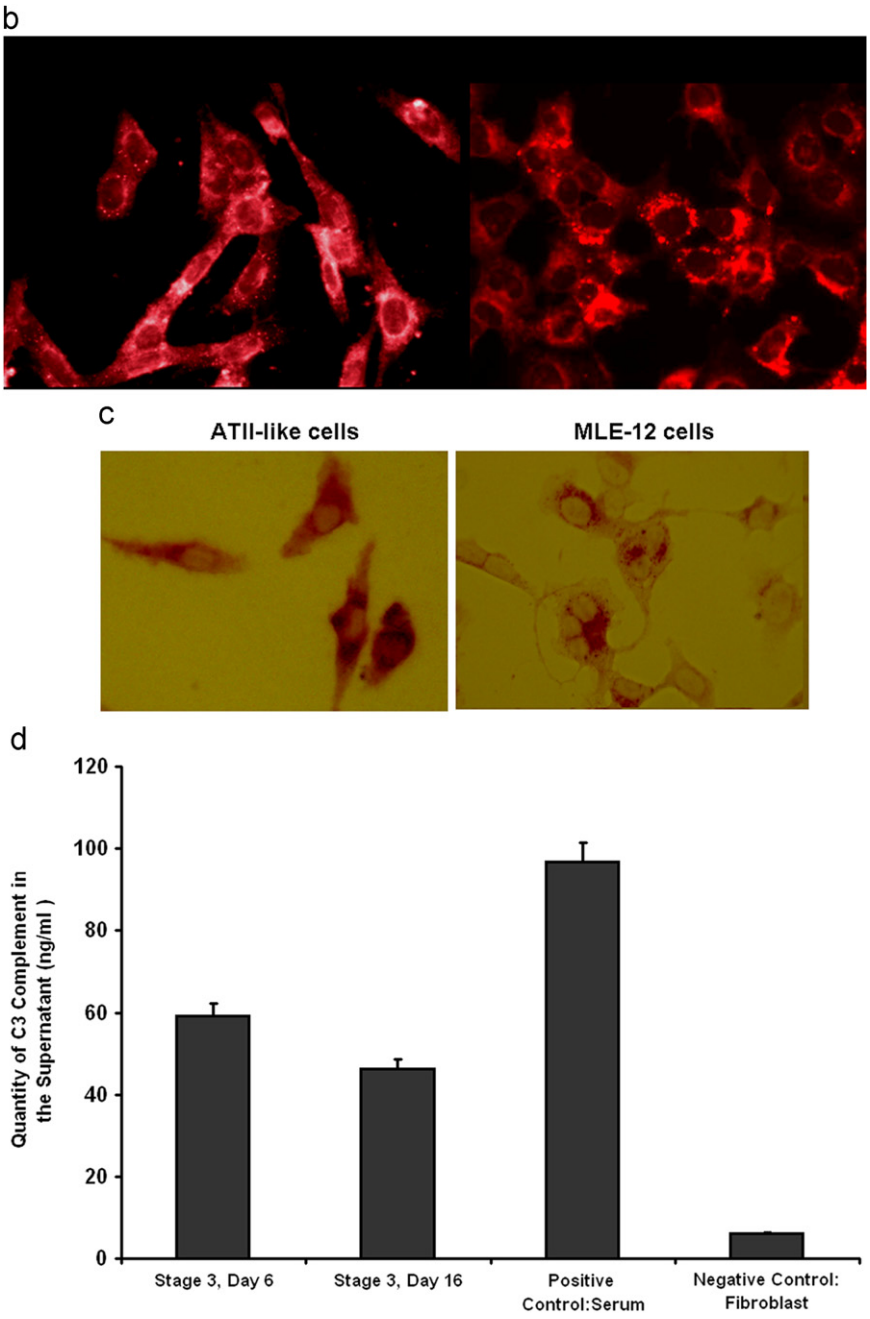

Fig. 2. (*continued*)

(integrin alpha V), both were highly expressed after 7 days of bleomycin treatment 8 μg/ml. This data suggests that the cells are potentially motile and can invade ECM from the basal membrane. Additionally, E-cadherin is directly influenced by the expression of Snai1 transcription factor, a master gene relating to EMT. During EMT progression, Snai1 suppresses E-cadherin gene expression. Upregulation of Snai1 and downregulation of E-cadherin was first observed in cells treated for 4 days: at higher doses of bleomycin cellular toxicity was observed and quantification of these protein expression levels was deemed unreliable. The expression of S100A4, a fibroblastic EMT marker seen in fibrotic diseases and cancer (Chen et al., 2001), is usually correlated with a-Sma gene expression levels. After 7 days of bleomycin treatment S100A4 was highly expressed although no upregulation was observed after 2 or 4 days of treatment. In contrast, a-Sma was upregulated early at Day 2, with 4 and 8 μg/ml bleomycin, with expression approximately 3 fold more than the untreated control. After 7 days of treatment, the a-Sma expression level was still elevated approximately three fold. Therefore, the co-expression of S100A4 and a-Sma after 7 days of 8 μg/ml bleomycin exposure generated EMT-derived fibroblasts. Additionally, Collagen1, an ECM protein that is directly correlated with EMT (Yamamoto et al., 2000), was highly upregulated after 4 days of treatment at all doses of bleomycin by immunostaining (Fig. 3f) and quantitative real time PCR analysis (Fig. 3g). No cellular toxicities were observed after 2 days of treatment; however, slight toxicity was observed at the highest bleomycin dose on Days 4 and 7 of treatment as measured by PI staining (data not shown).

The collective data indicates that bleomycin exposure at 8 μg/ml for 7 days induces the ATII cells to exhibit plasticity, transdifferentiate into a different cell type and undergo EMT.

3.3. Induction of EMT by TGF_{b1} and EGF

Various laboratories have shown that TGF_{b1} induces EMT in many organ systems including the lungs (Gal et al., 2008; Illman et al., 2006; Wang et al., 2008). It has also been shown that EGF enhances EMT when coupled with TGF_{b1} (Uttamsingh et al., 2008). In this study, we investigate the combined effects of TGF_{b1} and EGF on inducing EMT in ATII-like derived from iPS cells. EMT induction was achieved by treating Stage 3 Day 7 ATII-like cells with the following TGF_{b1}–EGF concentrations: 5 ng/ml TGF_{b1}+50 ng/ml EGF, 10 ng/ml TGF_{b1}+100 ng/ml EGF, and 20 ng/ml TGF_{b1}+200 ng/ml EGF, for 2, 4, and 7 days. The progression of EMT was assessed using the same tests as applied to EMT induced by bleomycin.

Morphological changes were already obvious after 2 days of treatment (Fig. 4a). The cells had flattened and elongated spindle-

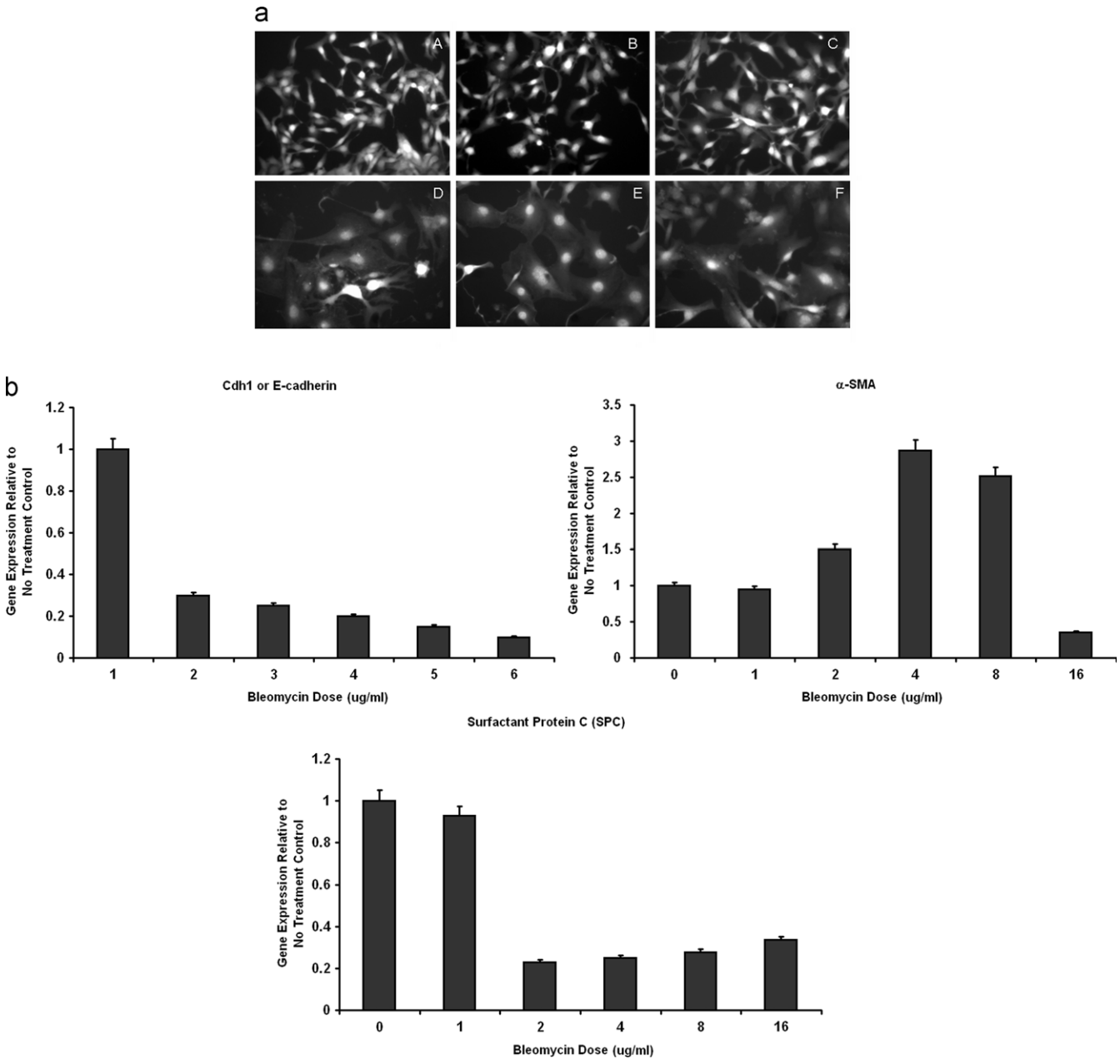

Fig. 3. (a) Morphology of Stage 3 Day 7 cells after four (4) days of bleomycin treatment. ((A) non-treated, (B) treated with 1 μg/ml bleomycin, (C) treated with 2 μg/ml bleomycin, (D) treated with 4 μg/ml bleomycin, (E) treated with 8 μg/ml bleomycin, (F) treated with 16 μg/ml bleomycin.) EMT-like phenotypic changes were observed as the bleomycin dose increased. Images were taken at 100× magnification. (b) Quantitative real time PCR (qRT-PCR) analysis of Stage 3 Day 7 ATII-like cells after four (4) days of bleomycin treatment showing mRNA expression of SPC and E-cadherin decreases while a-Sma gene expression increases as bleomycin dose increases. (c) Surfactant protein C expression of Stage 3 Day 7 ATII cells after four (4) days of bleomycin treatment showing decreasing SPC protein expression as the bleomycin dose increases ((A) non treated (100×), (B) treated with 1 μg/ml bleomycin (100×), (C) treated with 4 μg/ml bleomycin (200×), (D) treated with 16 μg/ml bleomycin (200×)). (d) Western blot analysis of Stage 3 Day 7 ATII-like cells after four (4) days of bleomycin treatment using E-cadherin and a-Sma. Protein expression of E-cadherin (a cell adhesion marker) decreases as bleomycin dose increases while myofibroblasts marker, a-Sma, increases indicating induction of ATII-like cells to EMT. (e) The EMT inducing condition using bleomycin is achieved by treatment of 8 μg/ml bleomycin exposed for seven days (7) on Stage 3 Day 7 ATII cells. Gene expression of EMT markers was quantified by quantitative real time PCR analysis. (f) Immunofluorescence staining of Col1 (an extracellular matrix usually upregulated in EMT) on Stage 3 Day 7 ATII-like cells after 4 days of treatment showing marked increase in protein expression as the bleomycin dose increases ((A) non-treated (100×), (B) treated with 2 μg/ml bleomycin (100×), (C) treated with 4 μg/ml bleomycin (100×), (D) treated with 8 μg/ml bleomycin (200×)). (g) Upregulation of Collagen 1 quantified by qRT-PCR on Stage 3 Day 7 ATII-like cells after 4 days of treatment.

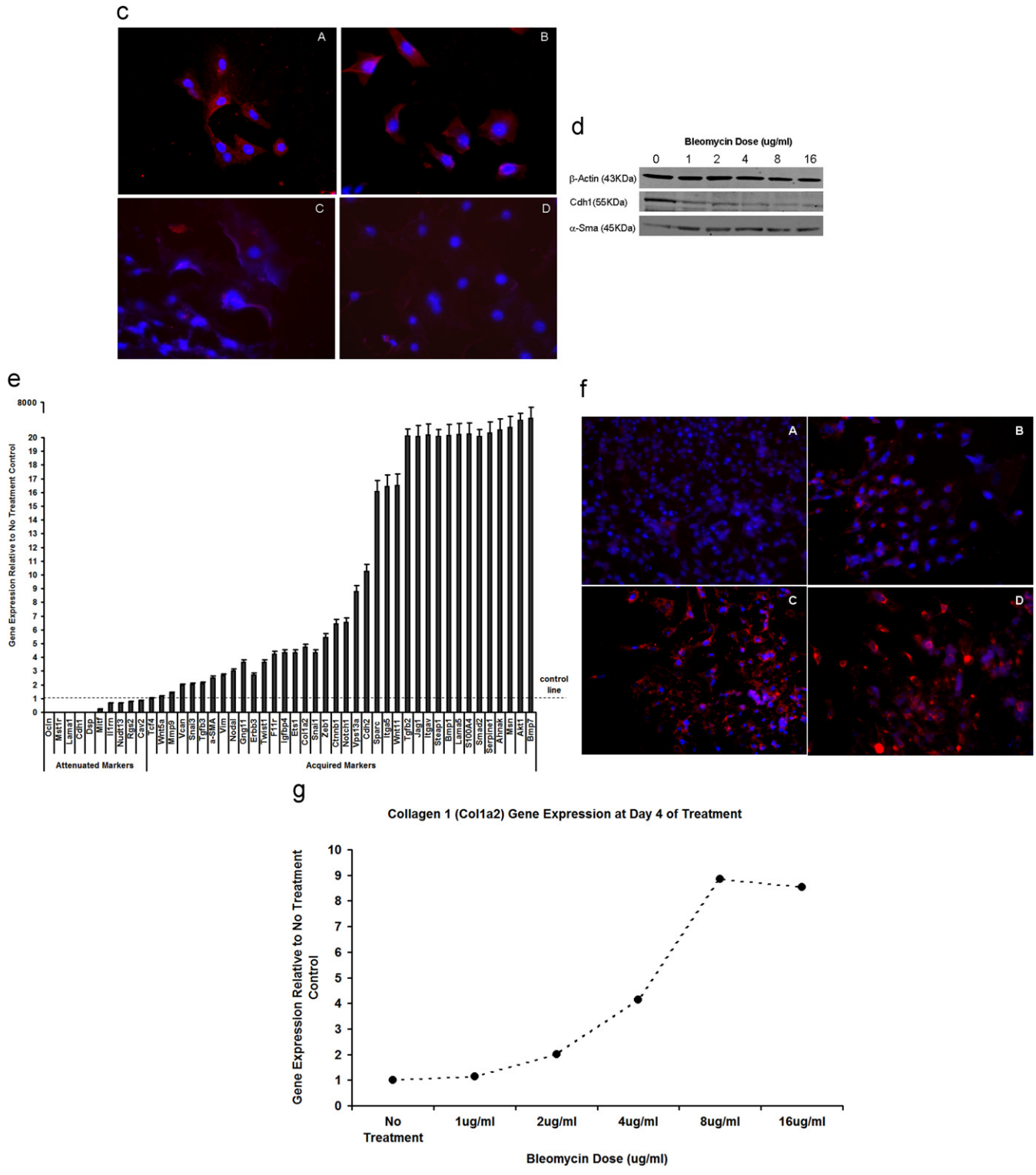

Fig. 3. (*continued*)

shaped features at 5 ng/ml TGF_{b1}+50 ng/ml EGF that became more pronounced as the dose increases. qRT-PCR analysis was performed and approximately 44% of the EMT markers, tested such as extracellular matrix markers (Col3, Col5, Ctnnb1, Gsc, Smad2, Snai1, Snai2 (Slug), and Twist1) and differentiation/development markers (Col1, Cdh2, Itga5, Itgav, Mmp2, Mmp3, Mmp9, and Timp1) were upregulated after only 2 days of treatment. Downregulation of E-cadherin also was already noted at this early stage of transdifferentiation by qRT-PCR analysis (Fig. 4e). More EMT markers were upregulated as the concentration of the growth factor cocktail increases for example, protein expression of SPC, Col1, and a-Sma was readily observed by immunofluorescence staining. SPC was highly expressed prior to treatment and slowly lost expression as the concentration of TGF_{b1}–EGF increases (Fig. 4c). Col1 protein expression is minimally expressed prior to exposure and increased its expression as the dose of TGF_{b1}–EGF increases (Fig. 4c). a-Sma expression was not profound by immunofluorecence staining (Fig. 4c) but showed 2 fold increase of

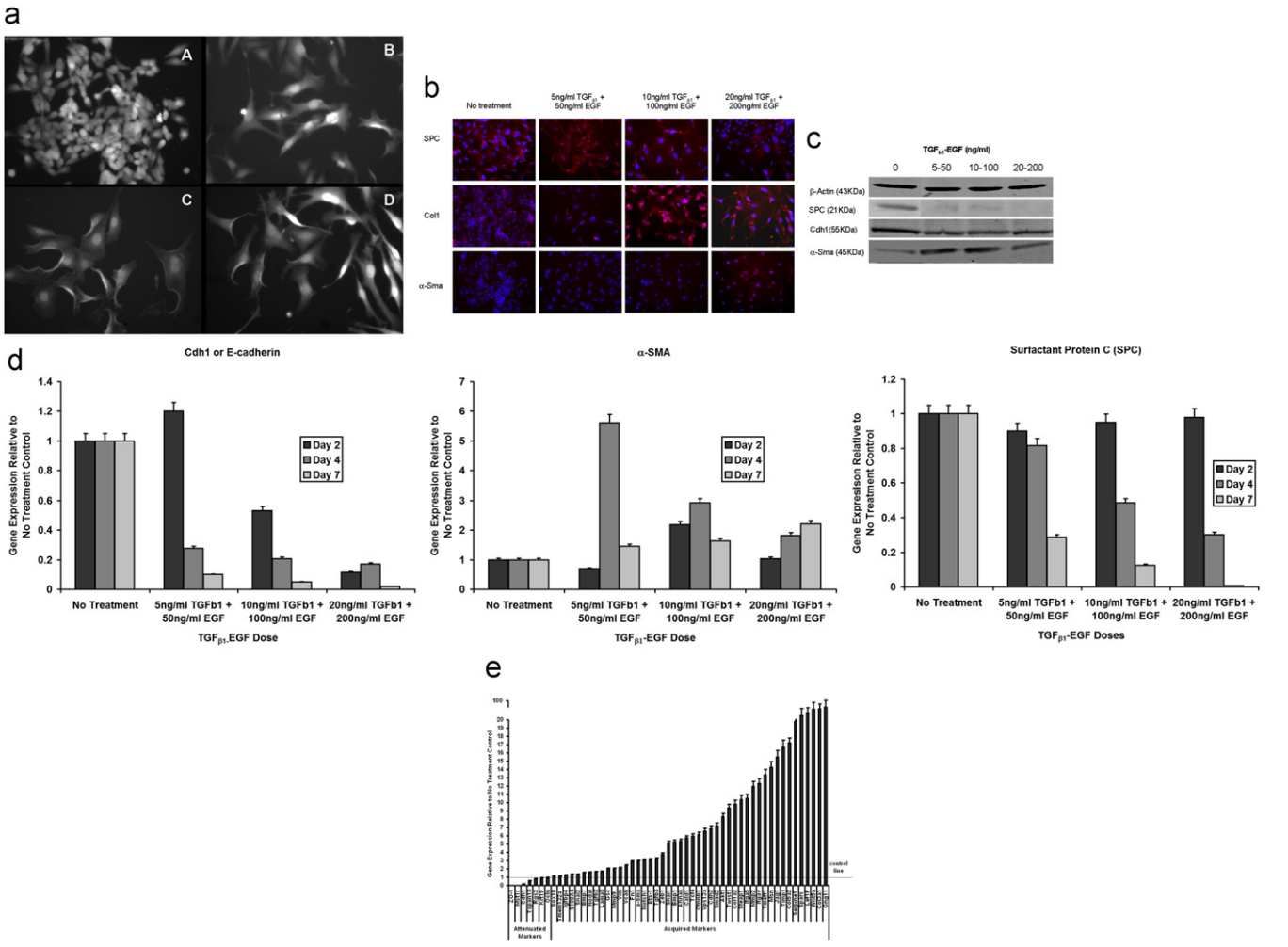

Fig. 4. (a) Morphology at Stage 3 Day 7 after two (2) days of TGF_{b1}–EGF treatment starting at day 5 ((A) non-treated (100 ×), (B) treated with 5 ng/ml TGF_{b1} + 50 ng/ml EGF (200 ×), (C) treated with 10 ng/ml TGF_{b1} + 100 ng/ml EGF (200 ×), (D) treated with 20 ng/ml TGF_{b1} + 200 ng/ml EGF (200 ×). At Day 2 of exposure, the effects of TGF_{b1}–EGF exposure on the ATII cells are robust. (b) Protein expression analysis by immunofluorescence staining at Stage 3 Day 7 ATII cells treated with TGF_{b1}–EGF for 2 days. SPC expression slowly decreases while Col1 increases as the dose of TGF_{b1}–EGF treatment increases. Images were taken at 100 × magnification. (c) Western blot analysis of Stage 3 Day 7 ATII-like cells after four (4) days of TGF_{b1}–EGF treatment showing decreasing SPC and E-cadherin protein expression while the expression of a-Sma increases. (d) Quantitative real time PCR analysis of Stage 3 Day 7 ATII-like cells TGF_{b1}–EGF treatment showing mRNA expression of Surfactant Protein C (SPC), a-Sma and E-cadherin. (e) The EMT inducing condition using TGF_{b1}–EGF is achieved by treatment of 10 ng/ml TGF_{b1} + 100 ng/ml EGF exposed for four days (4) on Stage 3 Day 7 ATII cells. Gene expression of EMT markers was quantified by quantitative real time PCR analysis.

mRNA expression at 10 ng/ml TGF_{b1} + 100 ng/ml EGF by qRT-PCR analysis (Fig. 4d). After 4 days of exposure, the cells have transformed robustly into a mesenchymal/fibroblastic phenotype feature (data not shown) and the qRT-PCR analysis shows that multiple EMT markers are highly upregulated (Fig. 4e). a-Sma is minimally expressed throughout by immunofluorecence staining (Fig. 4b) but is highly expressed in western blot analysis (Fig. 4c). E-cadherin was downregulated at all doses at this time point. Samples were obtained from Days 2, 4, and 7 of exposure at all doses and analyzed by qRT-PCR for EMT markers. SPC and E-cadherin were downregulated while a-Sma was upregulated as the dose increases. Other features of EMT such as cadherin switch, upregulation of integrin markers, S100A4, a-Sma and Col1 were also observed at all doses as early as Day 2 of TGF_{b1}–EGF exposure.

In summary, induction of EMT in ATII-like cells can be achieved after 2 days of exposure at either of the growth factor doses tested with a slight preference to 20 ng/ml TGF_{b1} + 200 ng/ml EGF dose due to morphology feature alterations (loss of cell to cell contact and fibroblastic morphology), qRT-PCR results and protein expression analysis although optimal condition to induce EMT is Day 4 of treatment with the 10 ng/ml TGF_{b1} + 100 ng/ml EGF dose. Prolonged exposure to TGF_{b1}–EGF cocktail (any dose at Day 7) is not a recommended culture condition due to poor cell morphology and increased presence of apoptotic cells.

4. Discussion

iPS cells have been recently used extensively as a cell source for differentiation into many discreet cell types of the body and to study their potential role in cellular therapy or to investigative the molecular basis of diseases (Alipio et al., 2010; Hanna et al., 2007; Xu et al., 2009). We document here that iPS cells can be used to generate ATII-like epithelial cells *in vitro* and that they undergo EMT when treated with the use of fibrosis stimulating agents such as bleomycin and TGF_{b1}–EGF. These observations suggest that the ATII-like cells can be used as an *in vitro* model to study pathophysiology of diseases involving fibrosis.

These ATII-like cells have the characteristics and features of ATII epithelial cells similar to the murine lung epithelial cell line MLE-12. The ATII-like cells have an epithelial phenotype with microphilopidia extensions, they express surfactant proteins, and

test positive for the presence of lamellar bodies and lipid deposits. The ATII-like cells mature slowly in culture and they retain their ATII-like features for a prolong period of time.

We evaluated the potential of ATII-like cells to be used as a model of lung fibrosis *in vitro* by treating them with fibrosis stimulation agents such as bleomycin and TGF_{b1}–EGF. Both bleomycin and TGF_{b1}–EGF readily induce EMT; however, the response times and the resulting phenotypic changes were not identical. ATII-like cells treated with bleomycin acquired a mesenchymal phenotype and express various proteins relating to EMT more slowly than growth factor treated cells, acquiring the most EMT features only after 7 days of exposure and at a concentration of 8 μg/ml bleomycin. E-cadherin, normally highly expressed in ATII cells, was significantly depleted only at high bleomycin dose and prolonged exposure time. Surfactant protein C, another specific marker for ATII cells, was significantly down-regulated. Concurrenty, bleomycin treated cells acquired several important EMT markers such as Col1, Snai1, Snai2, Vim, a-Sma, S100A4, and Twist1, further evidence that the ATII-like cells have indeed transformed into mesenchymal cells.

TGF_{b1} is involved in many physiological functions in the body such as tissue development and differentiation, extracellular matrix production and apoptosis. This protein also has been implicated as the root cause of EMT during injury, especially in the lungs. It is endogenously expressed in injured tissues as well as in patients suffering from fibrotic diseases such as idiopathic pulmonary fibrosis (IPF) and is a major EMT inducer (Illman et al., 2006; Uttamsingh et al., 2008; Wang et al., 2008). For TGF_{b1}–EGF treated cells *in vitro*, transdifferentiation occurred within the first two days in the treatment process, even at the lowest dose of the cocktail used (5 ng/ml TGF_{b1} + 50 ng/ml EGF). Full expression of EMT-related protein was observed by 4 days of treatment suggesting an involvement of a more robust signaling pathway (more likely a Smad dependent pathway due to enhanced Smad expression) and is consistent with observations from many laboratories using this fibrosis stimulating agent. The number of acquired EMT markers doubled compared to the ATII treated with bleomycin. Markers of differentiation and development (Col3, Col5, Ctnnb1, Jag1, Notch, Smad2, Tgfb3, Tmeff1, Twist1 and Vcan), cytoskeleton (Vim) and extracellular matrix (Cdh2, Col1, Itga5, Itgav, Mmp2, and Mmp9) were all enhanced in TGF_{b1}–EGF treated cells.

The data presented here strongly indicate that the ATII-like cells produced from skin fibroblast via an iPS cell intermediate can be utilized to study the molecular mechanism of EMT in lung cells *in vitro*. The study of the intracellular signaling pathways involved in both bleomycin-mediated or TGF_{b1}–EGF-mediated EMT will need to be examined and will hopefully lead to therapeutic targets for the amelioration of fibrosis in the lungs and other organs.

Disclosures

The authors indicate no potential conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.diff.2011.05.001.

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